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Androgen-induced relaxation of uterine myocytes is mediated by blockade of both Ca^{2+} flux and MLC phosphorylation.

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Abstract

Context: Uterine quiescence must be maintained until pregnancy reaches term. Premature activation of myometrial contractility leads to preterm labour and delivery.

Objective: To scrutinise the potential of androgens to relax the myometrium and the mechanism of their action.

Samples: A pregnancy-derived myometrial smooth muscle cell line (PHM1-41), myometrial strips prepared from tissues obtained from pregnant women [lean (n=9) and obese (n=6)] undergoing elective C-section at term and from non-pregnant C57BL/6 mice (n=5) were each utilised.

Design: The contraction of collagen-embedded PHM1-41s and the stretch-induced contraction of human and murine myometrial strips were assessed following incubation with testosterone (T), dihydrotestosterone (DHT) and T conjugated to BSA (TBSA). Intracellular calcium ([Ca^{2+}]) and phosphorylated myosin light chain (PMLC) concentrations were quantified in PHM1-41s using a Fluo-4 Ca^{2+} assay and in-cell Westerns (ICW) respectively.

Setting: University Research Institute.

Results: DHT and T, but not TBSA, impaired the contractile function of PHM1-41s and of human and murine myometrial strips. The response was rapid (observed within minutes), sustainable for up to 48 hours, and not abolished on knockdown of the androgen receptor (AR). DHT (100 μM) reduced the amplitude of lean strip contraction to 2%±2 of the pre-treatment value and T (100 μM) to 3.3%±1. These values for obese strips were 15%±6.7 and 11%±6.7 respectively. At the same doses, in murine strips, DHT reduced the amplitude to 4.8%±3 and T to 4.9%±3. DHT (50 μM) pre-treatment reduced the OXT-stimulated increase in [Ca^{2+}] ($p<0.0001$, n=6) and PMLC ($p<0.05$, n=5) in PHM1-41s.

Conclusion: Lipid soluble androgens could be developed as tocolytic agents for the treatment of preterm labour.

Introduction

Preterm birth (PTB), defined as birth before 37 weeks of pregnancy, accounts for 5-18% of all recorded births worldwide (1). Importantly, PTB is associated with long-term neurodevelopmental outcomes and an increased risk for respiratory and gastrointestinal complications in the offspring (2). The major obstetric precursor leading to PTB is spontaneous preterm labour, the outcome of preterm onset of regular myometrial contractions. The first line management of threatened PTB is initiation of tocolytic medications to suppress these contractions. Their mode of action is gene-transcription independent and involves rapid inhibition of key components in the contraction cascade, for example the oxytocin receptor (OXTR) and the various calcium (Ca^{2+}) channels (3). Tocolytics reduce the availability of intracellular Ca^{2+} ($[Ca^{2+}]$), prevent the phosphorylation of myosin light chain (MLC) and, thereby, the synchronised contraction of the myometrium. The currently used short-term tocolytic agents, such as Nifedipine, an L-type Ca^{2+} channel blocker, and OXTR antagonists, have high tocolytic efficacy in the short term, but their lack of longer term effect limits their effect on perinatal mortality (4,5). Conversely, magnesium sulfate - an inhibitor of MLC phosphorylation – which is the most commonly used tocolytic in the USA, is associated with maternal side effects and has low tocolytic efficacy (6).

Steroid hormones are currently the focus of much interest for PTB treatment and prevention. Prophylactic administration of vaginal progesterone (P) to pregnant women at high risk has been shown to reduce the rate of PTB by 50% (7). Our research group has previously demonstrated that exposure of spontaneously contracting myometrial strips to progesterone (P) resulted in a rapid (<30 minutes) reduction in the amplitude and integral of contraction, in line with P's well-established role in maintenance of pregnancy (8). In addition to P, one study reported that androgens in micromolar doses also relaxed human myometrial contractions *ex vivo* (9). We have recently reviewed all the evidence for a role of androgens in maintenance of pregnancy (10). Considering that a) tocolytics in current use delay delivery only by 24 hours to 7 days, b) P supplementation prevents only one-third of all recurrent PTBs and finally c) androgens produced by the placenta could be involved in the maintenance of pregnancy, we hypothesised that androgens should be investigated as novel PTB therapeutic agents. However, there is limited evidence on the efficacy of androgens, and the mechanism of action of androgens in preventing uterine contractions is poorly understood. Herein, we sought to address the effects of androgens on myometrial contractions and explore how they interact with the contractile apparatus. Specifically, we aimed to deduce a) whether T, dihydrotestosterone (DHT; non-aromatisable metabolite of T) and the cell-surface impermeable T (TBSA) inhibit the contraction of uterine myocytes *in vitro* and *ex vivo* in both human and mouse and b) to test the hypothesis that androgens prevent uterine contractions via reduction in the concentration of [Ca^{2+}] and, hence, reduction in the phosphorylation of MLC.

Materials and Methods

Human Tissue

Biopsies were obtained from the upper margin of the lower segment of myometrium from women undergoing elective caesarean section (ECS) as previously described (11) at the Simpson’s Centre for Reproductive Health at the Royal Infirmary of Edinburgh, following informed written consent. Ethics approval for recruitment of all pregnant women was granted by the West of Scotland Research Ethics Committee 4 (09/S0704/3) to the Edinburgh Reproductive Tissue BioBank. Biopsies were collected from lean (LN; 19<BMI<25) and obese (OB; BMI>25) women delivering at term (>37 weeks of gestation) prior to the onset of labour. Patients with twin pregnancies and pregnancy complications were excluded. The recovered biopsies were collected in ice-cold Rosewell Park Memorial Institute 1640 medium (RPMI; Gibco), rinsed in PBS and dissected into 2x2x15 mm strips parallel to the muscle fibre bundles.

Mouse Tissue

Experimental procedures were licensed (PPL 60/4241; PIL 60/13875) under the UK Home office Animals (Scientific Procedures) Act (1986). Murine uterine horns were harvested from 8-week old non-pregnant C57BL/6 mice supplied by Charles River (London, UK) and prepared into uterine strips (1 cm long each).

Human Uterine Myocytes

Pregnant human myometrial 1-41 (PHM1-41) cells were obtained from a single late-term pregnant donor as previously described (12). PHM1-41s were cultured as detailed elsewhere (13,14) with the exception that we used phenol red-free high-glucose Dulbecco's modified Eagle's medium (DMEM;

Lonza, UK). A PHM1-41 cell line in which the AR had been silenced (hAR-PHM1-41s) was produced using microRNA lentivirus. A scramble microRNA lentivirus (in which the AR remained active) was used as a negative control (Scr-PHM1-41s) as detailed in Supplemental Data and shown in **Supplemental Figure 2.**

Experimental compounds

DHT, T, Nifedipine, T3-(O-carboxymethyl)oxime:BSA (TBSA) were purchased from Sigma (Poole, UK) and oxytocin (OXT) from Alliance Pharmaceuticals (Chippenham, UK). DHT and T were reconstituted in ethanol (etOH), Nifedipine in DMSO and OXT was diluted in dH_2O. TBSA, with conjugation ratio T (30 molecules):BSA (1 molecule), was reconstituted in PBS. Anti-Phosphorylated Myosin Light Chain (PMLC) polyclonal antibody (Cell Signaling, UK) was used in 1:50, anti-α-Tubulin monoclonal antibody (Sigma) in 1:1000 and secondary antibodies 800CW and 680RD in 1:10000 (Li-Cor Biosciences, UK).

Organ bath

The assessment of myometrial contractility utilising organ bath is well established (8,13,15,16). Briefly, human myometrial and mouse uterine strips were attached by silk suture (Mersilk 3-0, Ethicon Inc) to a force transducer (ML0186/10 Panlab ADInstruments, UK) and stretched under passive resting tension (20 mN) in Krebs buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM Glucose, pH 7.4) equilibrated with 95% O_2-5% CO_2 at 37°C. Strips were allowed an equilibration period of 2 hours to develop spontaneous rhythmic contractile activity before addition of DHT or T in cumulative concentrations (10 μM to 100 μM) or TBSA (0.5 μM equivalent to 100 μM dose of T). Each treatment was applied for 30 minutes for human tissue and 10 minutes for mouse. Equivalent doses of vehicle (etOH or PBS) were applied; the minimum and maximum concentration of etOH used was 0.03% and 0.3% respectively. At the end of each experiment, the strips were stimulated with KCl (55 mM) and washed with fresh Krebs buffer to verify tissue viability/recovery. Data was recorded with LabChart 7 acquisition software (AD Instruments). The average frequency, peak amplitude and force integral (area under curve; A.U.C.) following each treatment were calculated for each strip as a percentage of its pre-treatment values.

Gel contraction assay

Cells were embedded in type I collagen in 24-well plates at 10^5 cells/well as previously described (13,14). Briefly, the collagen/cell suspension was allowed to polymerise and the gels were detached and incubated at 37°C for 24 and 48 hours with treatments prepared in 5% (v/v) charcoal-stripped fetal bovine serum (FBS) DMEM. Untreated or vehicle-treated cells developed a basal contraction, which manifested as a decrease in the gel area and was first evident 24 hours post detachment. The gels were photographed using a Leica MZ6 light microscope/camera (Mayfair, UK) at 0, 24 and 48 hours. Adobe Photoshop CS6 (CA, USA) was used to measure gel area. The measurement (pixels) for each gel area at 24 and 48 hours was reported as a percentage of the gel area at the 0-hour time point. The viability of cells in gels was assessed using CellTitre 96 AQueousOne Solution Cell Proliferation Assay kit (Promega, UK).

In-cell Western (ICW) blot analyses

Due to the rapid oscillations between the phosphorylated and dephosphorylated states of MLC and in order to accurately capture the cell transient contractile state, we utilised ICW, to quantify PMLC in PHM1-41s as described elsewhere (13,17). Briefly, cells were seeded into black-wall/optically clear-bottom tissue culture treated 96-well plates (PerkinElmer) to a concentration of 1.8×10^4 cells/well in charcoal-stripped 5% (v/v) FBS DMEM. Following application of treatments, cells were fixed in 3.7% (v/v) formaldehyde (Sigma) and incubated with primary and secondary antibodies. The plate was scanned using the Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences). The intensity of PMLC fluorescence was calculated relative to α-Tubulin in the same well.

Calcium assay

The BDTM Calcium Assay Kit (BD Biosciences) was employed to measure [Ca^{2+}] concentration in PHM1-41s. The assay was performed as described in Li et al (18). Briefly, PHM1-41s were seeded in white 96-well plates with a clear bottom (Perkin Elmer) in charcoal stripped 5% (v/v) FBS DMEM at a density of 3×10^4 cells/well. Following attachment, the cells were first incubated with the Ca^{2+} indicator, and then treated with DHT or vehicle (etOH). The plates were placed onto a fluorometric imaging plate reader (FLIRP)-NOVOstar (BMG Labtech, Germany) with built-in injectors. Prior to the injection of a compound, the basal cellular fluorescence, which denoted the concentration of

[Ca^{2+}], was recorded for 20 seconds using the MARS Data Analysis Software (BMG Labtech). Following injection, the changes in the fluorescence were recorded for 40 seconds. The readout was the highest fluorescence measurement recorded (peak) following injection and that was compared between treatments.

Statistics

All analysis was conducted with GraphPad Prism v6.0 (La Jolla, USA). For human and mouse, n represents the number of individual patients or mice. For cells studies, the n number denotes the number of times the experiment was repeated and the number of replicates per experiment is indicated in the figure legends. For statistical analysis, all percentage-presented data were arcsine-transformed. Data was analysed as indicated in the figure legends and presented as the mean ± SEM; $p<0.05$ was considered statistically significant.

Results

Androgens inhibit the contraction of uterine myocytes embedded in collagen gels

We set out to explore the effect of lipid soluble androgens DHT and T and of the cell-impermeable TBSA on the contraction of PHM1-41s cells. PHM1-41 cells were embedded in gels and incubated with vehicle (etOH), DHT or T (1 µM, 50 µM, 100 µM) or TBSA (0.5 µM) for 24 and 48 hours. Over time, vehicle gels developed a basal contraction resulting in a decrease in the gel area (**Figure 1A**). At 24 hours, the vehicle area was 77.2%±3.4 of the original (measured at 0 hours) (**Figure 1B**) and at 48 hours the area decreased to 65.2%±3.8 (**Figure 1C**). In contrast, the gel area of cells treated with DHT and T at 50 µM and 100 µM (**Figure 1A**), but not 1 µM, was significantly greater compared to the time-matched vehicle gel area, suggesting that both androgens prevented basal contraction. At 24 hours, the DHT (100 µM) gel area was 95.8%±1.2 ($p<0.0001$ vs vehicle) of the area recorded at 0 hours (**Figure 1B)** and 82%±6.4 ($p<0.05$ vs vehicle) at 48 hours (**Figure 1C**). For T (100 µM), these values were 94.9%±1.1 ($p<0.001$ vs vehicle) at 24 hours (**Figure 1B**) and 87.72%±5.5 ($p<0.001$ vs vehicle) at 48 hours (**Figure 1C**). TBSA treatment (**Figure 1D**) did not prevent basal contraction at 24 (**Figure 1E**) and 48 hours (**Figure 1F**), suggesting that the T-mediated inhibition of contraction is unlikely to be cell-surface receptor mediated. In addition, the finding that DHT (50 µM) prevented the basal contraction of PHM1-41s in which expression of the AR was silenced (hAR-PHM1-41s; **Figure**

1 G, H) suggested that AR is unlikely to be involved in the induction of relaxation by androgens. Finally, a viability assay ruled out the hypothesis that androgens at high micromolar doses induce cell death (**Figure 1I**). We conclude that long (>24 hours) exposure to lipid soluble androgens can inhibit uterine smooth muscle contraction *in vitro* via an AR-independent mechanism that is likely to be mediated by penetration through the cell membrane.

Androgens relax human and mouse uterine smooth muscle ex vivo

We examined the effect of short-term (<6 hours) exposure of androgens on spontaneous contractions of LN and OB human (**Figure 2**) and mouse (**Figure 3**) myometrium. Cumulative doses of DHT and T were applied onto human myometrial (**Figure 2A**) and murine uterine strips (**Figure 3A**) all contracting in organ bath chambers. Progressive significant reductions in average amplitude and A.U.C. were observed as the dose of T or DHT was increased from 10 μM to 100 μM for human (LN: **Figure 2B, D**; OB: **Figure 2F, H**) and murine (**Figure 3B, D**) tissue. Only at the 100 μM dose, the frequency of contraction significantly decreased following treatment with DHT and T for LN (**Figure 2C**), OB (**Figure 2G**) and murine (**Figure 2C**) tissue. In order to inform future *in vivo* experiments, we calculated the IC50 values of DHT and T on amplitude and A.U.C. of contraction (**Table 1)**. The IC50 values were not significantly different between the OB and LN groups and between human and mouse tissue. Contractions of myometrial strips were not affected by TBSA (0.5 μM) in human (LN: **Figure 2E,** OB: **Figure 2I**) or mouse (**Figure 3E**).

The organ bath studies combined with the gel contraction studies allowed the observation that lipid soluble androgens induce a rapid but sustained inhibition of uterine contractions.

Androgens inhibit MLC phosphorylation in uterine myocytes

Elevation in [Ca^{2+}] activates the Ca^{2+} sensor calmodulin, which binds to MLC kinase, activating MLC phosphorylation and subsequent contraction. We aimed to deduce whether DHT treatment prevented the phosphorylation of MLC (PMLC) in contracting PHM1-41s. OXT was utilised to stimulate contraction of collagen embedded-PHM1-41s. A 24- and 48-hour treatment with OXT enhanced contraction, which manifested as a decrease in gel area, with the area being smaller than that of vehicle (**Figure 4A**). After 24 hours (**Figure 4B**) the average vehicle gel area was 83.4%±6.9 of the original gel area (measured at 0 hours) and it was significantly different ($p<0.001$) when compared to the time-matched OXT gel area (66%±1.9). The co-treated OXT+DHT gel area was 82.8%±2.8 and significantly bigger than that the OXT gel area ($p<0.01$), demonstrating that DHT prevented the OXT-stimulated contraction (**Figure 4B**). The co-treated OXT+DHT gel area reduced to 78.2%±1.2 after 48 hours (**Figure 4C**) and was significantly different ($p<0.0001$) to the time-matched OXT gel area (42.2%±3.7).

To determine whether the effect of DHT involved blockade of MLC phosphorylation, we assessed the impact of DHT pre-treatment on PMLC concentration following acute (30 seconds) stimulation with OXT. Acute stimulation with OXT induced a dramatic increase ($p<0.001$) in the concentration of fluorescently-detected PMLC and a short (15 minutes) pre-incubation with DHT, but not vehicle, significantly ($p<0.05$) prevented the increase in PMLC following acute OXT (**Figure 4E**). Interestingly, pre-incubation with a Ca^{2+} channel blocker Nifedipine, prior to acute OXT, also significantly ($p<0.01$) prevented the increase in PMLC concentration (**Figure 4G).** We conclude that DHT inhibits PHM1-41s contraction via inhibition of MLC phosphorylation. The similarity between the actions of DHT and the L-type Ca^{2+} channel blocker Nifedipine with regards to prevention of MLC phosphorylation, contributes to the notion of an indirect effect of DHT on PMLC, potentially mediated via blockade of Ca^{2+} channels and subsequent decrease in [Ca^{2+}].

Androgens inhibit Ca^{2+} flux in uterine myocytes

We set to explore the hypothesis that DHT pre-treatment would prevent the increase in [Ca^{2+}] concentration in PHM1-41s. OXT was used to stimulate a rapid increase in [Ca^{2+}] concentration. Addition of OXT to untreated PHM1-41s induced an immediate 2-fold increase above baseline ($p<0.0001$) in the concentration of [Ca^{2+}] (**Figure 5B**). The effect of OXT on [Ca^{2+}] was examined following pre-treatment with either DHT or vehicle. DHT pre-treatment induced a dose-dependent reduction in the OXT-stimulated increase in [Ca^{2+}], which was significant when compared to the OXT-stimulated increase in [Ca^{2+}] in the vehicle pre-treated cells (**Figure 5B, C, D**). These data suggest that DHT blocks Ca^{2+} flux in uterine myocytes and impacts downstream MLC phosphorylation.

Discussion

A relaxant effect of androgens on smooth muscle contraction has been reported in different systems (19-23). Ten years ago, a single study demonstrated that various androgens, including DHT and T, relaxed human myometrial strips contracting under resting tension in organ bath chambers (9). The authors described the response as rapid (minutes), transcription independent (not prevented by protein synthesis inhibitors), achievable with pharmacological (micromolar) doses, and as reversible. Herein we show for the first time that a) only lipid soluble androgens (T, DHT) effectively relax obese and lean human and murine myometrial contractions, b) the response is immediate (minutes) but can be sustained for longer times (days) even in the presence of cell viability, c) the mechanism of relaxation is a reduction in the availability of [Ca^{2+}] concentration, which subsequently results in reduction of MLC phosphorylation in the uterine myocytes and, finally d) the mechanism of relaxation is AR-independent.

Other studies have reported the effects of sex hormones on [Ca^{2+}] and PMLC concentrations in other cell types and tissues. For example, DHT treatment of Fura-2-loaded isolated rat vas deferens cells blunted the KCl-induced elevation in [Ca^{2+}], while short incubation with estradiol (E2) inhibited the histamine-induced increase in [Ca^{2+}] in Fura-2-loaded airway smooth muscle (ASM) cells (24,25). These findings are in line with the inhibitory effect of DHT on OXT-stimulated increase in [Ca^{2+}] concentration in Fura-4-loaded PHM1-41s in our study. Consistent with our finding that DHT blunted the effect of OXT on PMLC, incubation with E2 and P in micromolar doses inhibited increases in PMLC in retinal epithelial and colon muscle cells (26,27).

It is reasonable to speculate that androgens restrict Ca^{2+} flux in uterine myocytes. Such an effect can be achieved either by physical interaction with Ca^{2+} channels or indirectly by interaction with molecules residing on the cell membrane, which are known to regulate Ca^{2+} channel activity (28). A physical interaction of androgens with Ca^{2+} channels has never been described but there is some evidence to support an indirect effect of androgens on Ca^{2+} channels. The antagonism of OXT by DHT observed in our study might suggest that androgens interact with the mechanism by which OXTR signalling activates capacitive and non-capacitive Ca^{2+} entry in PHM1-41s (29). The binding of OXT to OXTR, a G protein-coupled receptor, activates transmembrane receptor operated Ca^{2+} channels (ROCCs) to induce Ca^{2+} flux from the extracellular space into the cell but can also stimulate the IP3 cascade, which results in the activation of IP3 receptors on the sarcoplasmic reticulum (SR) and release of Ca^{2+} from the internal store into the cytoplasm (28,30). Therefore, it is plausible that DHT blocked either the ROCCs-associated pathway or the downstream activators of the IP3 pathway, which manifested as a decrease in total concentration of $[Ca^{2+}]$ in PHM1-41s. However, evidence from a coronary muscle study, where T failed to inhibit caffeine- and carbachol-induced (activators of IP3-pathway) Ca^{2+} release from the SR, suggests that androgens are likely to block the ROCCs-associated Ca^{2+} flux rather than the IP3 pathway (31). We hypothesise two mechanisms by which androgens could decrease the ROCCs-associated Ca^{2+} flux: a) Bind to a cell surface-associated binding protein that interacts with the OXTR and induce conformational changes to the receptor, which could result in impaired interaction of OXTR with the G-protein or b) overload the plasma membrane and change membrane fluidity, which could prevent the OXTR from interacting with the G-protein. Notably, if a membrane-initiated response were to mediate the effect of T in the myometrium, TBSA would be expected to inhibit the myometrial contractions in our study. However, TBSA did not induce relaxation, suggesting that the action of T is unlikely to be mediated via cell-surface receptors but requires penetration into, or through, the cell membrane. Therefore, it is possible that penetration of hydrophobic androgens into the negatively charged lipid bilayer altered the contractile function of PHM1-41s via impairment of cell membrane fluidity, which is known to affect active and passive transport of various molecules (32). The mechanism by which OXTR causes the opening of ROCCs is not clear (33), however, understanding this mechanism would help determine how androgens interact with the contractile cascades and inform whether they could be utilised as alternative tocolytics.

It is noteworthy that Nifedipine's uterorelaxant effect comes to prominence within 20 minutes of administration to pregnant women presenting with preterm contractions, and the impact of a single dose can last for up to 6 hours (34). The rapid response of myometrium to Nifedipine resembles the immediate (minutes) response to androgens observed in our study *ex vivo* in the term and possibly preterm (**Supplemental Figure 1**) myometrium. Adding to the similarity noted between the two responses, we showed that short incubations with DHT or Nifedipine each reduced the OXT-stimulated PMLC in PHM1-41s, suggesting that both compounds can rapidly manipulate components of the contractile apparatus.

With the aim of decreasing maternal and fetal side effects during tocolysis and delaying pregnancy until term, there is growing interest in the discovery and validation of alternative tocolytics. The benefits and harms of supplemental P, which inhibits human myometrial contraction with similar IC50s (16) to androgens in our study, are currently under investigation. Nifedipine, as well as other Ca^{2+} channel blockers, can cross the placenta and elicit adverse effects upon the fetus (3) but the placenta is known to possess mechanisms that inhibit the transport of androgens (35). In particular, the placenta can aromatise native androgens, such as T, to estrogens to protect the fetus from virilisation. A female fetus would only be in danger of virilisation if the androgen was administered during the masculinisation window, which is reported to exist during the first trimester of pregnancy (36). Conversely, animal studies have informed that maternal androgen excess is associated with the development of PCOS in the offspring (37). However, in the majority of these studies, androgen excess was achieved by a daily administration of non-aromatisable DHT in high concentrations from mid-gestation up to term (38,39). We believe it is unlikely that androgens will cause PCOS in female offspring, if given in native form for short periods to stop preterm-initiated contractions in the third trimester.

Further basic understanding of the dose response and the mechanism of action of androgens on uterine contractions are required to inform the design of preclinical studies on androgens as tocolytic agents. Notably, the IC50 values generated here could help design experiments whereby administration of DHT or T to existing mouse models of PTB (40) could be used to investigate if androgens can induce uterine relaxation. Such studies could contribute to the discovery of much needed novel preterm birth therapeutics.

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Figures and Table Legends

Figure 1: DHT and T, but not TBSA, inhibited the contraction of human myometrial cells embedded in collagen gels. PHM1-41s were embedded in collagen gels in 24-well plates and incubated with vehicle, DHT, T or TBSA for 24 and 48 hours. Over time, vehicle gels developed a basal contraction, which manifested as a decrease in the gel area (**A**). The gel area at each time point was measured and reported as a percentage of the original gel area. The post treatment percentages of the original gel area were compared to those of vehicle. DHT and T (50 μM and 100 μM) incubation for 24 (**B**) and 48 (**C**) hours significantly inhibited the basal contraction of PHM1-41s; *p<0.05, **p<0.01, ***p<0.001 compared to vehicle (etOH), n=7 (6 replicates). TBSA treatment at 0.5 μM (0.5 μM equivalent to 100 μM dose of T) did not inhibit the basal gel contraction after 24 (**E**) and 48 hours incubation (**F**); **p<0.01, ****p<0.0001, ns=non-significant compared to vehicle (etOH+PBS), n=5 (6 replicates). Silencing of AR in PHM1-41s did not prevent the effect of DHT (50 μM) on the basal contraction. 24-hour (**G**) and 48-hour (**H**) incubation with DHT (50 μM) induced a significantly smaller reduction in the gel area of wt PHM1-41s, scramble miR-infected (Scr-PHM1-41s; negative control) and hAR miR-infected (hAR-PHM1-41s) cells (i.e., with knock down of the AR); ***p<0.001, ****p<0.0001 comparison between vehicle and DHT groups, a=not significant: comparison with wt-PHM1-41s vehicle, b=not significant: comparison with Scr-PHM1-41s vehicle, c= not significant: comparison with wt-PHM1-41s-DHT group, d= not significant: comparison with Scr-PHM1-41s-DHT group, n=5 (6 replicates). **I:** Viability of PHM1-41 cells post incubation with DHT and T (100 μM) for 48 hours.

PHM1-41 cells were embedded in collagen gel and treated with DHT and T. Viability assay was performed on the gels 48 hours post treatment. Treatments with DHT and T did not affect viable cell number, which manifested as no change in cell metabolic activity; ns=non-significant compared to vehicle (etOH), n=4. Cell viability data were analysed using Kruskal-Wallis with Dunn's post-hoc test. Gel contraction data were analysed using one-way ANOVA with either Tukey's post-hoc test (**B, C, E, F**) or Sidak's multiple comparison test (**G, H**).

Figure 2: DHT and T, but not TBSA, rapidly relaxed spontaneous contractions of myometrium obtained from LN and OB women undergoing ECS at term. **A:** Representative recordings show the effect of DHT, T and TBSA on stretched-induced myometrial contractions of the LN group. Each contracting LN and OB myometrial strip was incubated with either cumulative doses (10 μM-100 μM) of vehicle, DHT or T, or with a single dose of TBSA (0.5 μM equivalent to 100 μM dose of T). Each dose was applied for 30 minutes. Concentration response curves were generated to show the effect of DHT, T and vehicle on average amplitude, frequency and A.U.C. of LN (**B-D**) and OB (**F-H**) myometrial contraction. For LN, the amplitude **(B)** and A.U.C. **(D)** of contraction decreased in a dose-dependent manner following either DHT or T; the decrease was significant at all doses tested. At 100 μM dose of DHT, the amplitude of contraction reduced to 2%±2 of the original value (**B**) and the A.U.C. to 4.5%±2 (**D**). T (100 μM) also reduced the amplitude of contraction to 3.3%±1.3 (**B**) and the A.U.C. to 15.8%±3.8 (**D**). The frequency **(C)** of contraction significantly decreased with the 100 μM dose of DHT and T ($p<0.0001$ compared to vehicle). For OB, the amplitude **(F)** and the A.U.C. **(H)** of contraction decreased in a dose-dependent manner following either DHT or T; the decrease was significant at all doses tested. At 100 μM, DHT reduced the amplitude to 15%±6 (**F**) and the A.U.C. to 4.3%±2.7 (**H**). At the same dose, T reduced the amplitude to 11%±6.7 (**F**) and the A.U.C. to 10%±5 (**H**). The frequency **(G)** of contraction significantly decreased only with the 100 μM dose of DHT and T ($p<0.01$ compared to vehicle). Data were analysed using one-way ANOVA with Tukey's post-hoc test. TBSA did not relax LN (**E**) or OB (**I**) human myometrial contractions; the effect of TBSA on the A.U.C. of contraction was no different to the effect induced by the vehicle (PBS). Data were analysed with two-tailed t-test; ns=non-significant, LN: n=5/1 strip per treatment, OB: n=6/1 strip per treatment.

Figure 3: DHT and T, but not TBSA, relaxed murine spontaneous uterine contractions. **A:** Representative recordings show the effect of DHT, T and TBSA on stretched-induced contractions of uterine horn strips. Each contracting strip was incubated with either cumulative doses (10 μM-100 μM) of vehicle, DHT or T, or with a single dose of TBSA (0.5 μM). Each dose was applied for 10 minutes. Concentration response curves were generated to show the effect of DHT, T and vehicle on average amplitude **(B)**, frequency **(C)** and A.U.C. **(D)** of contraction. The amplitude **(B)** and A.U.C. **(C)** of contraction were dose-dependently decreased; the decrease was significant at all doses tested. DHT (100 μM) reduced the amplitude to 4.8%±3 (**B**) and the A.U.C. to 10.4%±5 (**D**). T (100 μM) reduced the amplitude to 4.9%±3 (**B**) and the A.U.C to 4.8%±2.9 (**D**). Only the 100 μM dose of DHT significantly decreased the frequency of contraction ($p<0.001$ compared to vehicle). For T, the frequency was significantly reduced at both 80 μM ($p<0.001$ compared to vehicle) and 100 μM ($p<0.0001$ compared to vehicle) dose. Data were analysed using one-way ANOVA with Tukey's post-hoc test (n=5 mice/1 strip per treatment). **E:** TBSA did not inhibit murine uterine horn strip contraction; the effect of TBSA on the A.U.C. of contraction was no different to the effect induced by the vehicle (PBS). Data were analysed with two-tailed t-test; ns=non-significant, n=5 mice /1 strip per treatment.

Table 1: DHT and T IC50 values were generated from the concentration response curves for amplitude and A.U.C.

Figure 4: DHT treatment prevented the phosphorylation of MLC stimulated by OXT in human myometrial cells. The effect of DHT pre-treatment on OXT-stimulated contraction and OXT-stimulated MLC phosphorylation was investigated. PHM1-41s cells were embedded in collagen gels and incubated with vehicle (dH_2O + etOH), OXT (100 nM), DHT (50 μM) or OXT+DHT for 24 hours and 48 hours (**A**). The gel area was measured and reported as a percentage of the original gel area (0 hour time point). The OXT gel area was significantly smaller when compared to the vehicle gel area, however co-treatment with DHT+OXT prevented the OXT alone-induced effect on the gel area at 24 (**B**) and 48 hours (**C**); ***$p<0.001$, ****$p<0.0001$ comparison between OXT and vehicle, ##$p<0.01$, ####$p<0.0001$ comparison between OXT and OXT+DHT, n=5 (6 replicates). **D:** PHM1-41s were seeded into 96-well plates and either directly exposed to acute (30 seconds) treatment with vehicle

(H_2O) or OXT (100 nM), or initially pre-treated (15 minutes) with vehicle (etOH) or DHT (50 μM) and then stimulated with acute OXT. **E:** The concentration of PMLC was significantly higher in the wells following acute OXT compared to the PMLC in the wells treated with the acute vehicle; ###p<0.001, n=5 (6 replicates). The concentration of PMLC in the vehicle pre-treated cells was higher compared to the concentration of PMLC in the DHT pre-treated cells when both were exposed to acute OXT; *p<0.05, n=5 (triplicate). **F:** PHM1-41 cells were either directly exposed to acute vehicle (H_2O) or OXT (100 nM) or first pre-treated (15 minutes) with vehicle (DMSO) or Nifedipine (50 μM) and then exposed to acute OXT. **G:** The concentration of PMLC in the DMSO pre-treated cells was higher compared to the concentration of PMLC in the Nifedipine pre-treated cells when both were exposed to acute OXT; ####p<0.0001 comparison between acute OXT and acute vehicle, **p<0.01 comparison between Nifedipine+OXT and vehicle+OXT, n=5 (triplicate). Data were analysed using one-way ANOVA with Tukey's post-hoc test.

Figure 5: DHT treatment prevented the rapid increase in [Ca^{2+}] concentration stimulated by OXT in human myometrial cells. **A:** Cells were seeded into 96-well plates and either not treated or treated with vehicle (etOH) or DHT (10 minutes) and then injected with OXT (10 nM). The injection of OXT to untreated wells rapidly increased the concentration of [Ca^{2+}] above baseline (red plot). The DHT pre-treatment (10 minutes) significantly reduced the response to OXT injection. OXT injection to vehicle (etOH) pre-treated wells increased the concentration of [Ca^{2+}] above baseline significantly more than to DHT (**B:** 300 nM, **C:** 800 nM, **D:** 50 μM) pre-treated wells; *p<0.05, **p<0.01, ****p<0.0001 comparison between the groups vehicle (etOH)+OXT and DHT+OXT, ###p<0.001, ####p<0.0001 comparison between the groups vehicle (H_2O) and OXT, n=6 (4 replicates). Data were analysed using one-way ANOVA with Tukey's post-hoc test.